Sulphonamide Carbonic Anhydrase Inhibitors

Studies with Special Reference to their Ocular Effects

by

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This review is based on experimental work by the author, as presented in the following communications, which will be referred to by Roman numerals as designated below.

I. WISTRAND, P., The effect of carbonic anhydrase inhibitor on intra-ocular pressure with observations on the pharmacology of acetazolamide in the rabbit. *Acta pharmacol. et toxicol. 16,* 171 (1959).

II. WISTRAND, P. J., RAWLS, J. A., JR. & MAREN, T. H., Sulphonamide carbonic anhydrase inhibitors and intra-ocular pressure in rabbits. A comparison between *in vitro* and *in vivo* activities based on tissue distributions and physical and chemical properties of nine compounds. *Acta pharmacol. et toxicol. 17,* 337 (1961).

III. WISTRAND, P., Local action of the carbonic anhydrase inhibitor, acetazolamide, on the intraocular pressure in cats. *Acta pharmacol. et toxicol. 14,* 27 (1957).

IV. LINNÉR, E. & WISTRAND, P., The initial drop of the intraocular pressure following intravenous administration of acetazolamide in man. *Acta Ophthalmol. 37,* 209 (1959).

V. AURICCHIO, G. & WISTRAND, P., Failure of carbonic anhydrase inhibition by acetazolamide to alter tonicity of the aqueous humour. *Acta physiol. scand. 44,* 118 (1958).

VI. WISTRAND, P., NECHAY, B. R. & MAREN, T. H., Effects of carbonic anhydrase inhibition on cerebrospinal and intraocular fluids in the dog. *Acta pharmacol. et toxicol. 17,* 315 (1961).

Introduction

A comprehensive review of the pharmacology of carbonic anhydrase inhibitors covering the literature through 1955 is given by Berliner &

Orloff (1956). A short review of the history of these inhibitors, leading to the present studies is given below.

The carbonic anhydrase inhibitors of sulphonamide type are almost unique in that practically all of their observed effects on the whole animal can be ascribed to the inhibition of one single enzyme, carbonic anhydrase.

They have all been deliberately fashioned to block reversibly this enzyme, which was presumed to have an important function concerned with the cellular handling of hydrogen and bicarbonate ions. The history of the drugs has been an instructive sequence of clinical observations, physiological and biochemical studies and attempts to synthesize more potent congeners.

Shortly after the introduction of sulphanilamide as a chemotherapeutic agent, it was observed in patients (Southworth, 1937) that the drug also induced metabolic acidosis, later found to be associated with an increased urinary excretion of bicarbonate. A couple of years later Mann & Keilin (1940) demonstrated that various sulphonamides with an unsubstituted sulphonamide group, like sulphanilamide, exerted a marked inhibitory effect on carbonic anhydrase *in vitro*. Soon thereafter Davenport & Wilhelmi (1941) reported that this enzyme was present in renal tubular cells. The following year Höber (1942) observed that sulphonamides cause prompt alkalinization of frog urine provided again that the sulphonamide group is free. Höber therefore concluded that renal carbonic anhydrase in some way was involved in the tubular reabsorption of bicarbonate and that sulphonamides by inhibiting the enzyme induce the excretion of alkaline urine. Subsequent experimental studies and theories by Pitts *et al.* (see Pitts, 1960) on the mechanism of acidification of the urine and the dependence of these theories on carbonic anhydrase led Schwartz (1949) to try sulphanilamide as a diuretic in patients with cardiac oedema. The promising results seen in these patients stimulated Roblin & Clapp (1949) to synthetize a series of highly active inhibitors of carbonic anhydrase. This culminated in the finding of acetazolamide (WHO), first tested on the dog kidney by Berliner *et al.* (1951). Acetazolamide was the first answer to the hope of finding a clinically useful and orally effective diuretic.

As early as 1935 Roughton had clearly defined the function of the enzyme in the red cells. At about the same time as the part played by carbonic anhydrase in the kidney began to be realized, theories were put forward which assumed that the enzyme also played a part in the function of the following organs where it had been found: gastric mucosa, pancreas, C.N.S., salivary glands and lens (cf. van Goor, 1948). However, theories on the function of carbonic anhydrase in other secretory organs were not proposed until around 1950.

In the eye Kinsey (1950) demonstrated a considerable excess of

bicarbonate ions in the rabbit aqueous humour and he ascribed to this ion the role of being the primarily secreted one. Friedenwald (1949) has suggested that the hydroxyl ions are actively transported into the aqueous humour. In analogy to other secretory organs it was then natural to assume carbonic anhydrase to have a function also with regard to the bicarbonate secretion of the aqueous humour. This assumption led to a search for and the finding of the enzyme in the iris and ciliary processes (Wistrand, 1951). Trials of carbonic anhydrase inhibitors on aqueous humour secretion with the hope of finding agents that lowered the intraocular pressure by reducing aqueous inflow were made in several laboratories. This was to introduce a new principle in the treatment of glaucoma.

In the eye *p*-toluenesulphonamide (Wistrand, 1954) and sulphanilamide (Kinsey, personal communication) were tested with disappointing results. As in other organs an unequivocal effect on the secretion was not observed until the appearance of acetazolamide, which was soon shown significantly to reduce the intraocular pressure in both man (Becker, 1954) and rabbits (Wistrand, 1954, Grant & Trotter, 1954).

The similarity of the aqueous humour and cerebrospinal fluid also suggested the trial of the effect of acetazolamide on the formation of the latter fluid (Tschirgi, 1954). However, in this case a theory involving the enzyme had not been a forerunner of the trial.

Since then many other organ functions have been tested after carbonic anhydrase inhibition. The fluid volume and total electrolyte output of the organs known to contain carbonic anhydrase have been found to be reduced, provided an inhibitor potent enough was used.

The pharmacodynamic actions of the new inhibitors on these organs attest to the validity of the early theories on the importance of carbonic anhydrase for maximal acid-base work of the cell. The usefulness of these drugs as research tools is therefore apparent. However, the use of these agents has not revealed the more intimate role of carbonic anhydrase.

According to one main theory, the enzyme is involved in the *direct* provision of hydrogen or bicarbonate ions (from carbonic acid) to the exchange mechanism of the cell. This theory has been proposed for the renal tubular cells by Pitts *et al.* (cf. Pitts, 1960) and for the ciliary epithelial cells by Becker (1959). Another main theory states that the role of the enzyme is an *indirect* one, namely the maintenance of the efficacy of the intracellular bicarbonate buffer system during the transfer of hydrogen or hydroxyl ions (from other sources than carbonic acid) out of the cells. This theory has been proposed for the gastric mucosa (Davies & Roughton, 1940) and for the ciliary cells (Friedenwald, 1955).

Purpose and Plan of Investigation

The experiments presented here had the purpose of contributing to the knowledge of the effects of carbonic anhydrase inhibitors in the intact animal.

Therapeutically these agents are most widely used in the treatment of glaucoma; by reducing the aqueous humour flow they are capable of lowering the intraocular pressure. Besides having important clinical uses they are favorite tools for studying the dynamics of the aqueous humour in order to gain further knowledge about the mechanism of its secretion.

Also for other reasons the eye was chosen as the target organ for recording the effects of these inhibitors. This Institute has long experience in aqueous humour dynamics. In contrast with most secretions the rate of aqueous humour flow is normally very stable. The measurement of the intraocular pressure is technically relatively easy and can be performed on a great number of animals (the pressure is directly related to the rate of aqueous secretion provided certain criteria are fulfilled, as discussed in paper I). Moreover, samples of the secreted fluid, the aqueous humour, can readily be obtained.

Tissue Distribution, Activity *in vitro* and *in vivo* and Physico-chemical Properties of Various Inhibitors

A great obstacle to the efforts to shed light on the more detailed effects of these drugs was overcome by the introduction by Maren, Ash & Bailey (1954) of a method for determining the inhibitors in body tissues. The fate and distribution in the body of these agents was thus open to study. In spite of the introduction of this method as early as six years ago very few reports on the effects of these drugs in relation to their distribution have appeared. Also a comparison of several inhibitors, in order to explain their different effects on whole animals, has not been available. Papers I, II and VI were therefore devoted to such studies; *in vivo* effects as recorded on the intraocular pressure were correlated with the *in vitro* activity, disposition and physico-chemical characteristics of various inhibitors.

The lowering of the intraocular pressure after systemic administration of carbonic anhydrase inhibitors is due solely to inhibition of the enzyme and most probably of that part of the enzyme occurring in the secretory cells of the ciliary processes. This conclusion is based on the following results. The similarity between the dose-response curves among the drugs (II) indicates that these compounds lower the eye

pressure by a similar mode of action. Moreover, the agreement between the *in vitro* activities and the *in vivo* activities among these structurally dissimilar sulphonamides (II) favours the concept that inhibition of carbonic anhydrase is the ultimate cause of reduction in intraocular pressure. The only marked exceptions were the almost totally ionised compounds, with pKa's below 4.2. These were only weakly active *in vivo*. Since these ionic species have very high activity in the cell-free *in vitro* system (II) it is tentatively concluded that their low activity *in vivo* is due to lack of access to the intracellular enzyme which in turn is a consequence of their low lipid solubilities. However, once within the cell both the ionised and, somewhat less certainly, the undissociated forms of the inhibitors probably inhibit the enzyme. This is made probable from the knowledge that *in vitro* a change of the ionisation status of the inhibitor does not considerably alter its inhibitor constant.

The inhibitor found most potent *in vitro,* ethoxzolamide (NND) was about 5 times more active than acetazolamide, the best known drug. The weakest inhibitor, sulphanilamide (NFN) was approximately 250 times less potent *in vitro* than acetazolamide; other tested inhibitors ranged between these extremes. For those having a pKa of 6.7 or more and an ether/saline partition coefficient larger than 0.04, these relative potencies also held *in vivo* (II). The *in vivo* effects were determined on the intraocular pressure 30 minutes after different doses given as intravenous injections.

The *in vivo* activities were found to agree reasonably well with the *in vitro* activities whether the former were derived from the doses or the plasma concentrations necessary for a fixed degree of response (II). The data were insufficient to decide which of the two moities of the drug in plasma, free or protein bound, was most closely related to the response.

All inhibitors seemed to give the same maximal effect, some 30–40% lowering of the outflow pressure, provided the doses were large enough. For acetazolamide single i.v. injections of doses between 1–120 mg/kg gave a dose-response relationship with doses above 10 mg/kg showing the maximal response (I). The magnitude of the pressure drop was directly related to the plasma concentration (I).

The duration of the eye-pressure response was dependent on the amount of inhibitor in plasma. Provided an effective concentration in plasma was maintained, the eye pressures remained constantly low as tested for several hours (I, II).

The onset of the effects on eye pressure was very rapid, as tested in rabbit (I), cat (III), dog (VI) and man (IV). The effect was observed within 2 minutes after the injection and reached its maximum within approximately 15 minutes. The effect seemed to be independent of the initial pressure in normal, ganglionic blocked or

anaesthetized rabbits (I). The onset of the effect of the more slowly penetrating drugs with low lipid solubilities remains to be studied. With acetazolamide there was such a short delay that almost instantaneous effects on the secretory process have to be assumed.

The rate of entrance of acetazolamide into the ciliary processes was rapid and so was the diappearance of the drug from this tissue (I). The concentration curves in the plasma and ciliary processes ran almost parallel after a single i.v. injection of acetazolamide. The rapidity by which this drug enters or leaves the ciliary processes and the plasma may have something to do with the fact, that topically administered acetazolamide has no effect on eye pressure (Wistrand, 1954, and many others). It is possible that the continuous washing effected by the drug-free plasma keeps the drug concentration in the processes low.

No marked difference in the ability to enter the ciliary processes was detected among acetazolamide, methazolamide (NND) and CL-11366 (numerical designation used by the American Cyanamid Co.) (II). The first two drugs have very similar physical chemical characteristics, methazolamide being somewhat more lipid soluble. The last agent has a low pKa, 3.2, and a very low lipid solubility. This bewildering finding, equal distributions in spite of differences in lipid solubilities, is probably explained by the rapid equal distribution of these different drugs in a relatively large extracellular portion of the ciliary processes. The distribution of these inhibitors within the actual secretory cells of the ciliary processes remains to be studied.

However, the ability to penetrate into the aqueous humours varied among these three drugs (II). Acetazolamide and methazolamide, in particular, rapidly entered the aqueous humours where they reached concentrations almost equal to the concentrations of free diffusible drug in plasma. In contrast, only very low concentrations of the totally ionised CL11366 were achieved in the anterior and particularly so in the posterior aqeuous humour.

When plasma concentrations were kept constant, at steady state, the concentrations of both acetazolamide and CL11366 were higher in the anterior aqueous humour as compared to the posterior aqueous humour. This shows that additional amounts of these drugs enter the anterior chamber by diffusion from the iris vessels. An active transport in the direction aqueous humour → blood (Becker, B., personal communication) by the epithelium lining the posterior chamber could augment the difference between the chambers.

The distribution of acetazolamide in other ocular tissues were studied during 5 hours of a constantly maintained plasma level (I). The build-up of concentrations in the cornea followed closely that in the anterior aqueous humour. Only relatively small amounts of the drug were detectable in the whole lens and vitrous body.

The distribution of these inhibitors in the red cells was also investigated (I, II, VI). The red cells are of particular interest, since they contain large amounts of carbonic anhydrase, the inhibition of which affects the CO_2 transport in the body (see Cain & Otis 1960). This in turn might influence the secretory functions of various organs. All inhibitors tested entered the red cell, methazolamide being the fastest. All of them were partly found as a strongly bound form, the concentration of which was markedly similar among the inhibitors, about 15 μg/ml (II). This concentration of bound drug within the red cells was independent of the plasma levels within a large range as tested for acetazolamide, methazolamide and CL11366 (II, VI). The bound acetazolamide was found to be very slowly cleared from the red cells (I).

The bound fractions of acetazolamide and methazolamide in the red cells are not related to the effects on the eye pressure (I), and are probably also devoid of respiratory effects (VI). These latter effects instead appear to be due to the inhibition of the enzyme by the easily diffusible component of the inhibitor within the red cells (VI).

For acetazolamide it was found that this diffusible component rapidly equilibrates with the non-protein-bound fraction of the drug in plasma. This was presumably true also for methazolamide and the highly ionised CL11366 (II) and CL13475 (unpublished observation).

Among the different inhibitors a comparison of plasma–protein binding, plasma half-lives and ionisation did not reveal any unequivocal relationships between these parameters (II).

Effects of Carbonic Anhydrase Inhibition on Cerebrospinal fluid (CFS) Pressure in the Dog

The similarities between the aqueous humour and CSF prompted similar studies on this latter fluid. CSF pressure was measured simultaneously with eye pressure in the dog; a comparison between the responses to carbonic anhydrase inhibition is given in paper VI.

A rapidly established concentration of the carbonic anhydrase inhibitors acetazolamide and methazolamide in the blood caused an immediate (20 sec) rise (30 to 180%) in the CSF pressure in anaesthetized dogs. A concentration of approximately 10^{-4} M/l in the red cell gave close to maximal response. The pressure response was not related to the amount of "bound" inhibitor within the red cells but to that of free inhibitor in equilibrium with carbonic anhydrase. Inhibition of this enzyme elevates the peripheral venous and tissue pCO_2. This suddenly increases the intracranial vascular bed causing the observed plethysmographic CSF pressure effects. The pressure curves

returned exponentially to their initial values in spite of continuous carbonic anhydrase inhibition. Slowly established high concentrations of the inhibitors in plasma and red cells, e.g. after oral administration, had no effect on CSF pressure. This is probably explained by the fact that in this case the process of striving for equilibrium keeps pace with the slowly dilating vascular bed.

No certain lowering of the CSF pressure was observed in these dogs during 1, 6 or 24 hours after the administration of the inhibitor. This was probably due to the fact that our anaesthetized dogs initially had comparatively low and scattered pressures approaching those within the veins.

In this paper (VI) the problem of determining the CO_2 equilibria of the blood during carbonic anhydrase inhibition is discussed. CO_2 equilibria derived by the Henderson–Hasselbalch equation from pH and total CO_2 are misleading, since in the body the bicarbonate buffer system is not in equilibrium, which, however, is essential for valid application of the equation. Application of this equation during carbonic anhydrase inhibition slightly underestimates the pCO_2 of venous blood, whereas it highly overestimates the corresponding values for the arterial blood (Cain & Otis, 1960). The extent of the error introduced by using the equation depends on what blood is used. Due to circulation-time effects, during carbonic anhydrase inhibition the values of pH, pCO_2 and HCO_3^- in the blood taken from the large vessels are probably quite different from those of the blood percolating through the secretory organs such as the ciliary processes and choroid plexus.

Do These Inhibitors Act Locally on the Secretory Organs?

Höber (1942) was the first to show that the effect of sulphanilamide on the perfused isolated frog kidney was local. Janowitz & Hollander (1954) also demonstrated the inhibiting effect of topically administrated unsubstituted sulphonamides on gastric secretion.

Since, following the systemic administration of carbonic anhydrase inhibitors, a "mixed acidosis" develops due to the red cell and kidney effect, it is reasonable to assume that this acidosis might be the cause of the eye-pressure effect, particularly since we know that both respiratory and metabolic acidosis are associated with a lowered eye pressure (Wistrand & Maren, 1960).

Some authors believe this to be the sole explanation (cf. Duke-Elder 1957). However, considering the presence of the enzyme in the ciliary processes and its assumed role in aqueous-humour secretion, it was found desirable to look for evidence for a direct effect on intraocular pressure. Acetazolamide and a control substance were infused in small doses into one respective carotid artery. On the inhibitor side

a more rapid and pronounced fall of the intraocular pressure was observed (III). The effect is thus local and could be due to either one (or both) of two possibilities, i.e. the inhibition of the enzyme in the ciliary secretory cells or in the red cells percolating through the ciliary processes. The poor effect of CL11366 and CL13475 on intraocular pressure (II) argues in favor of the former alternative being the most important. These highly ionised sompounds are very active inhibitors when tested on the enzyme *in vitro* and are known to penetrate into the red cells (II).

Some Chemical Aspects of Carbonic Anhydrase Inhibition

The effects of carbonic anhydrase inhibition on the rate of secretion of aqueous humours with different compositions were studied from various angles in paper IV, V and VI.

Man has an excess (relative to plasma water) of chloride and a deficit of bicarbonate in the aqueous humour. In the rabbit an excess of bicarbonate and a deficit of chloride is found (cf. Davson, 1956).

In man and rabbit carbonic anhydrase inhibition reduces considerably the concentrations (relative to plasma water) of the anions present in excess, chloride and bicarbonate, respectively (cf. Becker, 1959). In the dog, however, neither the total CO_2 nor Cl^- in the posterior and anterior aqueous humours were significantly lowered one hour after injection of 50 mg/kg i.v. of methazolamide (VI).

In paper IV and VI it was shown that the intraocular pressure in humans and dogs responded immediately to carbonic anhydrase inhibition as in the rabbit (I). The reasons for the different electrolyte responses to carbonic anhydrase inhibition are not understood, although the suggestion has been made that the ion primarily secreted is either Cl^- or HCO_3^-, dependent on the species (cf. Becker, 1959). Be that as it may, the pressure drop after carbonic anhydrase inhibition has the same characteristics in species having aqueous humours of different initial electrolyte composition. Moreover, the pressure drop occurs in the dog without a significant alteration in distribution of the measured ions between plasma and aqueous humour. It would thus appear that perhaps the basic secretory mechanisms in the three species are not so different as the ion distributions seem to indicate. Perhaps these distributions are the results of secondary processes superimposed on a common basic mechanism.

In paper V the effect of carbonic anhydrase inhibition on the composition of the aqueous humour as revealed by the osmotic pressure was investigated. A widely accepted theory (Kinsey, 1950) concerning the formation of aqueous humour postulates that the osmotic pressure

difference of about 100 mm Hg between the aqueous and the plasma is the principal force driving the flow of aqueous humour. Therefore it was of interest to see whether the changed composition of the rabbit aqueous humour following the administration of acetazolamide implies a reduction in the osmotic pressure difference between aqueous humour and plasma. The normally present hypertonicity (of about 4–6 mOsm/kg water) in both anterior and posterior aqueous humour was found not to be affected by systemic administration of acetazolamide. This finding cast doubt on the whole concept that osmotic forces are of importance for the transport of water into the aqueous humour. The problem was taken up by Auricchio & Bárány (1959). These authors found that the mobility of water across the blood–aqueous humour barrier was altogether too small to explain the normal flow of aqueous humour as a consequence of the existing osmotic pressure difference between the bulk of the aqueous humour and the blood. However, Auricchio & Bárány stressed the dangers of directly applying results of *in vitro* measurements of osmotic pressure to *in vivo* conditions and the matter is still not settled.

Concluding Remarks

Although studies of intact organs have contributed to our knowledge of the effects of carbonic anhydrase inhibitors, these studies have not revealed their more intimate mode of action. For this, further work on the cellular and enzymatic level is necessary. Thus there is need for more data on the relationship between the observed effects and the degree of inhibition of the enzyme within the intact cell. Present estimates (cf. Berliner & Orloff, 1956) of the degree of enzyme inhibition within an organ or cell after the administration of an inhibitor are based on measurements *in vitro*. Since, for technical reasons, such measurements are made on dilute tissue homogenates at low temperature, the extension of *in vitro* values to *in vivo* conditions is uncertain. If a highly irreversible inhibitor were available, the uncertainties of the estimate of the enzyme inhibition *in vivo* would be reduced, since the association between the enzyme and the inhibitor would not be so sensitive to the dilution effects *in vitro*. However, the direct measurement of the degree of inhibition in the intact cell would be still better. This was accomplished by Keilin & Mann in 1941 for the erythrocyte. For a better understanding of the effects of carbonic anhydrase inhibition on the cell, we need, moreover, to know better the cellular location of the enzyme. Datta & Shepard (1959) found the major portion of enzyme activity to be localized in the soluble fraction of rat liver and kidney tissue, obtained by differential centrifugation technique. There is also need for studies of the effect of the

inhibition on the performance of individual cells. A really thorough understanding of the effects, however, will probably have to await the time when the detailed mechanisms of the acid-base work of the cell are known.

Analyses of the type of inhibition, whether competitive or non-competitive, reversible or irreversible, are scarce. Sulphanilamide has been found to give a non-competitive type of inhibition (Davis, 1959). Such studies might give a better insight into the relationship between chemical structure and activities of these inhibitors.

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